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Granulocyte oxidative activity after thermal injury

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Background. Alterations in granulocyte function after thermal injury have been described. We have serially studied the level of granulocyte cytosolic peroxidase activity in 23 thermally injured patients during the first 6 weeks after injury. The patients' mean age and burn size were 35.1 ± 15.7 years and $41.6\% \pm 16.8\%$ (range, 18% to 88%), respectively. Fourteen patients had concomitant inhalation injury, and the overall mortality rate was 4.3%.

Methods. Purified granulocytes were obtained from peripheral blood after red cell lysis and Ficoll-Hypaque (Pharmacia Inc., Piscataway, N.J.) gradient separation. Cells were loaded with dichlorofluorescein diacetate, and baseline fluorescence was measured by flow cytometry. After phorbol myristate acetate stimulation, fluorescence was measured again. Cells from unburned normal subjects were used as daily controls.

Results. The data are expressed as percent of stimulated control granulocyte fluorescence. Unstimulated patient granulocytes demonstrated a significantly higher baseline activity than did unstimulated controls (22.9% vs 15.4%; $p < 0.05$). Mean fluorescence from stimulated granulocytes was 11.4% of the control values ($p < 0.05$).

Conclusions. Granulocytes from thermally injured patients exhibited a baseline increase in cytosolic oxidase activity, suggesting *in vivo* activation and a greater than normal oxidase activity after *in vitro* stimulation. (SURGERY 1992;112:860-5.)

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ALTHOUGH MICROBIAL INFECTION continues to be the leading cause of death in thermally injured patients, the exact mechanisms that lead to this increased susceptibility to infection remain to be elucidated. Historically, granulocytes from thermally injured patients were thought to be functionally impaired. Previous reports documenting defects in chemotaxis, phagocytosis, bactericidal capacity, and superoxide and hydrogen peroxide production¹⁻⁸ have been interpreted as indicating that granulocytes from thermally injured patients were dysfunctional. However, more recent investigations of

these cells have indicated that although *ex vivo* granulocyte migration and chemotaxis are abnormal, phagocytosis, bactericidal capacity, and oxidative burst are normal.⁹ Defects in chemotaxis have recently been attributed to an initial *in vivo* activation of white cells presumably by endotoxin, various cytokines, or complement activation byproducts.^{9, 10} This *in vivo* activation may result in enhanced granulocyte oxidative function. Conversely, early activation may lead to degranulation and exhaustion of cellular activities, such as the oxidative burst, that would result in subsequent abnormal cellular function. The current studies were undertaken to define more specifically the effect of burn injury on the oxidative burst potential of granulocytes as related to burn size and postburn day.

MATERIAL AND METHODS

Granulocytes from 23 burned patients from the U.S. Army Institute of Surgical Research were studied serially for up to 6 weeks after injury. All patients were admitted to the hospital within 48 hours of injury and underwent uneventful resuscitation. All patients were resuscitated with lactated Ringer's solution and the

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Table I. Patient population

Age (yr)	35.1 \pm 15.7 (21-89)
TBSA (%)	41.6 \pm 16.8 (18-88)
Inhalation injury (n [%])	14/23 (61)
Sex (M/F) (n)	20/3
Mortality (n [%])	1/23 (4.3)

Data are means \pm SD (range).
TBSA, Total body surface area.

modified Brooke formula. Topical wound care consisted of alternating silver sulfadiazine and mafenide acetate. Enteral feedings with a standard formula (Osmolyte), enriched with additional medium-chain triglycerides and protein, were begun between postburn days 3 and 5. No patient received ω_3 fatty acid supplementation. Burn wound excision was begun on postburn days 3 to 5 and continued as donor sites were available for wound closure. Granulocyte function tests were obtained as long as the patient had open wounds involving more than 15% of the total body surface. Patients were excluded from further study if they had evidence of sepsis during this study period. Patients treated with lymphokines or corticosteroids were excluded from this study. Granulocytes from one of 10 healthy laboratory personnel were used each day as controls for comparison purposes. In all, 275 control samples were run to establish normal values.

In vitro testing. Complete blood counts were obtained every Monday and Thursday from each active patient at 6 AM, before wound manipulation or surgery. An average of 10 samples (range, 1 to 12) was obtained from each patient during the first 6 postburn weeks. On the day of blood sampling, granulocytic cells were isolated from heparinized whole blood by Ficoll-Hypaque gradients. Whole blood (8 ml) was diluted with 16 ml Hanks' balanced salt solution (HBSS), and 6 ml of the diluted mixture was layered over 4 ml Ficoll-Hypaque in each of four 15 ml plastic conic centrifuge tubes. The gradients were spun at 200 *g* for 20 minutes. Cells passing through the gradient were recovered from the cell pellet, recombined in a 50 ml plastic conic centrifuge tube, and reconstituted in HBSS. The cells were spun at 2250 *g* for 10 minutes and the white cells were forced to the top of the pellet by the more dense red cells under the force of the centrifugation, forming a buffy coat. After aspiration of the supernatant, approximately 3 ml of the buffy coat was removed and placed in a new 50 ml plastic conic centrifuge tube. Contaminating red cells were removed by hypotonic lysis. Distilled water (20 ml) was added to the buffy coat cells during agitation of the sample on a vortex mixer. After 20 seconds, 20 ml

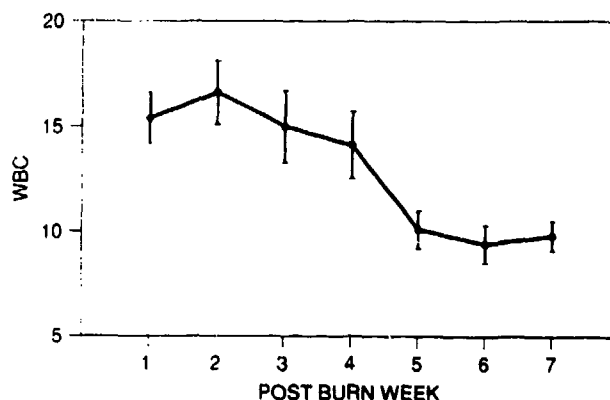


Fig. 1. Total white blood cell (WBC) count. All patients demonstrated mild leukocytosis that persisted through fourth postburn week and eventually returned to normal values.

hypertonic (2X) HBSS was added, the cells were centrifuged at 200 *g* for 10 minutes, and the supernatant was removed. The unlysed cells were suspended in 2 ml HBSS and transferred to a 15 ml conic centrifuge tube. A second lysis was performed by adding 4 ml distilled water for 20 seconds after which 4 ml 2X hypertonic HBSS was added to restore isotonicity. The cells were washed and resuspended at a concentration of 1×10^6 cells/ml in 1 ml barbital buffer, pH 7.25. Then 2',7'-dichlorofluorescein diacetate (DCF-DA; 5 μ mol/L final concentration) was added to each sample and incubated for 20 minutes at 37° C to allow DCF-DA to enter the cells. DCF-DA easily permeates the cells where the acetyl groups are hydrolyzed to create 2',7'-dichlorofluorescein (DCF). DCF is too polar to pass through the plasma membrane and is trapped effectively within the cell. When DCF is oxidized by peroxide, it becomes highly fluorescent, and the measurement of this fluorescence serves as an index of cytosolic peroxidative activity (oxidative burst). Cell fluorescence was measured by flow cytometry. The mean fluorescence of 10,000 cells was determined for each data point. After an initial fluorescence measurement, cells were incubated for 20 minutes with and without phorbol myristate acetate (PMA; 700 ng/ml) as stimulant. Measurements were recorded as log fluorescence and converted mathematically to linear fluorescence for group comparison. Patient cell responses were compared with values obtained from granulocytes from normal volunteers.

Statistical analysis. Differences between groups of patients were analyzed by analysis of variance adjusted for repeated measures with post hoc testing when appropriate with the BMDP statistical package (BMDP Statistical Software, University of California Press, Berkeley, Calif.).

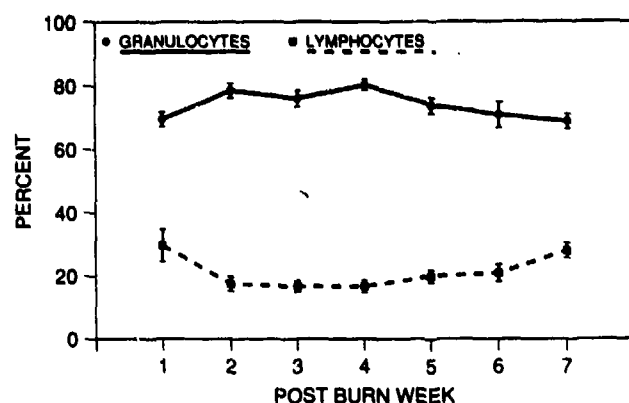


Fig. 2. White blood cell differential counts. Observed leukocytosis was characterized by granulocytosis and relative lymphopenia that resolved during patients' postburn course.

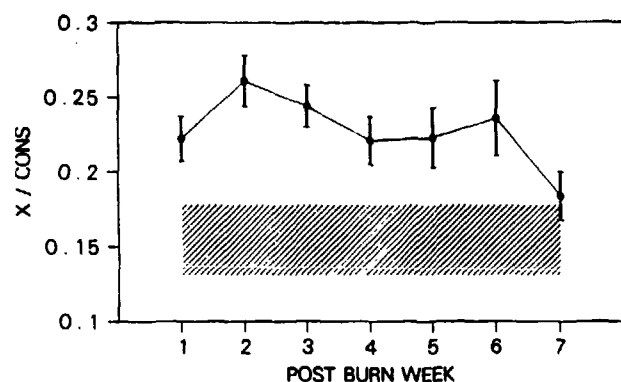


Fig. 3. Unstimulated patient fluorescence for all patients by postburn week. Data are depicted as x/cons (see text). Cross-hatched area represents 95% confidence limits of CONUR.

RESULTS

Patient population. Twenty-three patients with a mean age of 35.1 years and a mean burn size of 41.6% were studied (Table I). Fourteen of the patients (61%) were diagnosed as having inhalation injury by bronchoscopy or xenon 133 scan. One patient died, for a mortality rate of 4.3%.

Blood count data. All patients originally demonstrated mild leukocytosis that persisted until the fifth postburn week (Fig. 1). This leukocytosis was characterized by granulocytosis and relative lymphopenia that persisted throughout the course of the study (Fig. 2).

DCF oxidation. The mean linear fluorescence of unstimulated granulocytes from control subjects was 1883.7 ± 135 (mean \pm SEM). Stimulation with PMA increased mean linear fluorescence to $12,184 \pm 706$. The variance of the fluorescence for unstimulated and stimulated granulocytes from the controls was greater

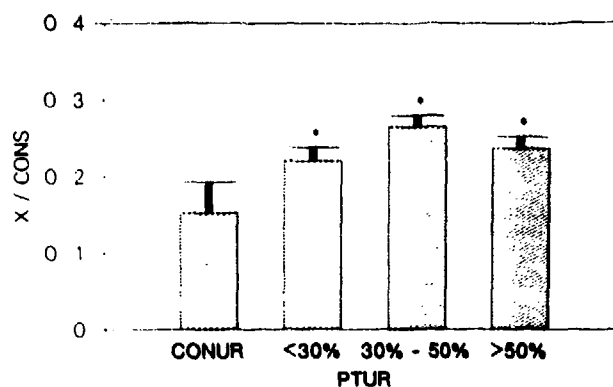


Fig. 4. Unstimulated patient fluorescence ratios for postburn days 0 to 21. Data are represented as mean \pm SEM. Numbers of observations in each patient group were 27, 43, and 38, respectively. All three patient groups demonstrated significant increase in unstimulated fluorescence compared with normal laboratory controls. Data are depicted as x/cons (see text). * $p < 0.05$ compared with CONUR.

between control subjects than the repeated measures made on each control subject over time. Thus to standardize the data for comparison purposes, the mean linear fluorescence for the unstimulated granulocytes of control subjects, as well as the unstimulated and stimulated granulocytes of the patients, was divided by the mean linear fluorescence for the stimulated granulocytes of laboratory control subjects. Thus for control subjects, two ratios are obtainable, CONUR and CONSR ($x/\text{cons} = \text{conu}/\text{cons}$ or cons/cons). CONUR is the ratio of the mean fluorescence of unstimulated granulocytes from control subjects to that of stimulated granulocytes from control subjects. Unstimulated granulocytes of laboratory control subjects demonstrated $15.4\% \pm 1.0\%$ fluorescence of the stimulated cells. The value for CONSR is always 1. PTUR and PTSR refer to the ratios of the mean linear fluorescence values for unstimulated and stimulated granulocytes of patients to those of stimulated control cells. Oxidative burst was calculated by subtracting the mean linear fluorescence of the unstimulated cells from the mean linear fluorescence of the stimulated cells and expressed as a percentage of that difference in control subjects.

Examination of all patient data from postburn days 0 to 42 reveals a mean PTUR of 0.229 ± 0.0067 , a value that is significantly higher than the value for granulocytes of control subjects (0.154 ± 0.01) ($p < 0.05$). This indicates a baseline increase in cytochrome oxidase activity before in vitro stimulation with PMA. This baseline increase in unstimulated granulocyte cytosolic oxidase activity was noted for the first 6

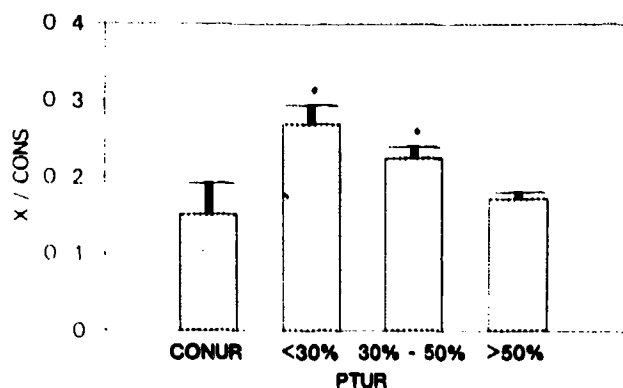


Fig. 5. Unstimulated patient fluorescence ratios for postburn days 22 to 42. Patients with burns less than 50% of total body surface area had unstimulated fluorescent ratios that remained significantly elevated compared with control values. Patients with larger burns demonstrated normal fluorescence ratios. Data are depicted as x/cons (see text). Number of observations was 36, 40, and 40, respectively, for each patient group. * $p < 0.05$ compared with CONUR.

weeks, indicating a persistent *in vivo* activation of the granulocytes (Fig. 3). PTSR, or the ratio for stimulated patient cells, was 1.14 ± 0.032 compared with a normal value of 1.0 ($p < 0.05$). These values were significantly different, indicating an increase in stimulated *in vitro* cytosolic peroxidase activity in neutrophils from burned patients. The mean oxidative burst for patient cells was 106.9% of control values.

In an attempt to define the effect of burn size and postburn day on the observed changes in cytosolic peroxidase activity, the patients were segregated according to burn size and postburn day of study. The patients were arbitrarily segregated into three groups according to burn size: 0% to 29% ($n = 7$), 30% to 50% ($n = 9$), and greater than 50% ($n = 7$) of total body surface area burn. Analysis of the unstimulated granulocyte fluorescence data when segregated by patient burn size and postburn day revealed a persistent prestimulation (*in vitro*) increase in cytosolic oxidase activity. All three groups of patients had a significant increase in unstimulated fluorescence compared with normal laboratory controls for the first 21 postburn days (Fig. 4). After 21 days, the fluorescence of unstimulated cells remained significantly elevated in the two groups of patients with burns less than 50% of total body surface area but returned to normal levels in patients with larger burns (Fig. 5). This paradoxical finding may be an indication that the bone marrow of patients with large burns is producing larger numbers of functionally immature cells. In addition, the patients with smaller burns remaining in the study after 21 days represent a subset

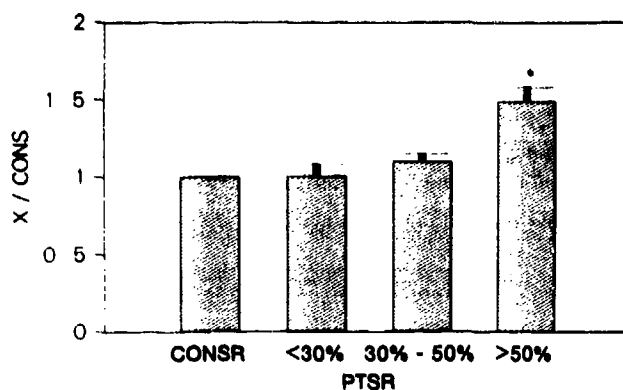


Fig. 6. Stimulated patient fluorescence ratios for postburn days 0 to 21. Stimulated fluorescence was statistically elevated in patients with burns greater than 50% of total body surface area compared with control values. Patients with smaller burns demonstrated normal stimulated cell fluorescence. Numbers of observations were 27, 43, and 38, respectively, for each patient group. Data are depicted as x/cons (see text). * $p < 0.05$.

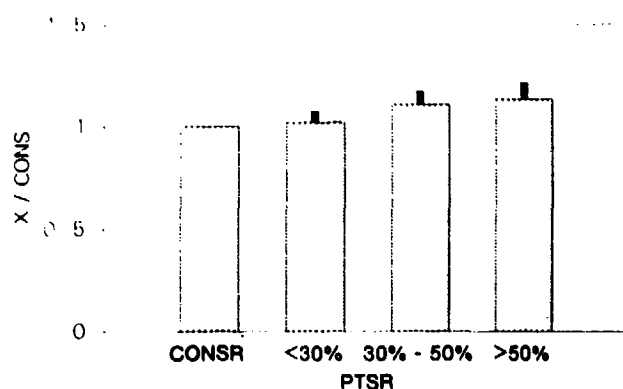


Fig. 7. Stimulated patient fluorescence ratios for postburn days 22 to 42. Stimulated fluorescent ratios returned to normal in all patient groups during this period. Numbers of observations were 36, 40, and 40, respectively, for each patient group. Data are depicted as x/cons (see text).

of patients who had complications such as pulmonary failure or pneumonia, which precluded wound closure. Thus this group of patients should not be construed as being representative of patients with smaller burns who had no complications at a point in time greater than 3 weeks after injury.

The fluorescence of stimulated cells was normal in patients with burns less than 50% of total body surface area for the first 21 days but was elevated significantly in the group with large burns (Fig. 6). The granulocyte oxidative burst was 147% of normal for this latter group. After postburn day 21, stimulated fluorescence was normal in all groups (Fig. 7).

DISCUSSION

This study documents a significant burn size-related increase in both resting and stimulated cytosolic oxidative function in granulocytes from thermally injured patients. This increase in unstimulated activity is consistent with what some investigators have termed an in vivo activation of neutrophils, presumably caused by the injury per se. The increase in stimulated activity compared with normal controls suggests that neutrophils from burned patients have an increased oxidative burst potential.

The concept of in vivo activation of neutrophils after thermal injury is well supported. Moore et al.¹⁰ studied neutrophil activation in seven thermally injured patients and found that the neutrophil expression of the complement opsonin receptors for both CR1 and CR3 was increased for up to 50 days after injury. This increase in receptor expression correlated with a defect in chemotaxis in response to zymosan-activated serum, suggesting that C5A was responsible for the systemic neutrophil activation. Further work by that same group examined polymorphonuclear neutrophil (PMN) function in 22 thermally injured patients.⁹ The investigators again found a marked increase in expression of CR1 and CR3 receptors. This finding was attributed to early postinjury exposure to endotoxin and elevated plasma complement products in the later postinjury phase. In addition, PMN phagocytosis and percent killing of *Staphylococcus aureus* was normal, which did not support earlier claims of general depression of neutrophil function after thermal injury. This marked and sustained increase in neutrophil expression of the complement receptors CR1 and CR3 was always observed as a monophasic peak, which suggested that all cells had been activated in vivo. Endotoxemia has been documented in the first 24 hours after thermal injury, with the systemic levels of endotoxemia related to burn size.¹¹ Activation of the complement system through the alternative pathway occurs after thermal injury in both murine models and humans. Prior decompensation with cobra venom factor ameliorates this finding and results in a decrease in pulmonary neutrophil aggregation.¹²⁻¹⁴

Maderazo et al.¹⁵ reported on a series of 46 patients with blunt trauma in whom they found a substantial increase in resting PMN hydrogen peroxide production compared with controls. This difference was accentuated when the PMNs were stimulated in vitro with opsonified zymosan particles. The spontaneous hydrogen peroxide production by the neutrophils of patients who had sustained trauma was similar to the hydrogen peroxide production by normal PMNs that were preexposed to a chemoattractant, such as zymosan-activated

normal serum. This further supports the concept of in vivo activation of PMNs by complement products, endotoxin, or various cytokines.

Dobke et al.¹⁶ reported that in a group of severely burned patients, resting neutrophil O₂ uptake was increased significantly compared with normal controls, a finding similar to ours. Stimulation of patient cells with opsonified zymosan resulted in a smaller increase in oxygen consumption than that seen in control cells. The authors suggest that this indicates a form of in vivo metabolic exhaustion. Our findings do not support this hypothesis because PMA-stimulated patient granulocytes exhibited a normal or supranormal oxidative burst, indicating an increase in cytosolic oxidative activity. This difference may be related in part to the different stimuli used. Dobke et al. used opsonized zymosan, a C3b receptor-mediated stimulus, whereas we used PMA, a non-surface receptor-mediated stimulus.

The functional consequences of systemic neutrophil activation remain to be elucidated. However, as hypothesized by Davis et al.⁹ and Moore et al.,¹⁰ if the activator is a specific chemotaxin, the chemotaxin receptors on the cell surface will be decreased as they are internalized. This would result in the inability of the cell to respond to further challenges with that same chemotaxin. A second, and perhaps more important, deleterious side effect of in vivo activation is the potential for neutrophil autooxidative damage as a result of this increased cytosolic oxidative potential. In the same group of 46 patients with blunt trauma, Maderazo et al.¹⁵ demonstrated a decrease in microtubular function by using the concanavalin A capping assay. Their results indicated that oxidant-induced microtubular dysfunction could lead directly to PMN chemotactic dysfunction. Finally, the CR3 receptor plays a role in cell adhesiveness. Thus increased expression may result in an increase in PMN attachment to endothelium, which may then be damaged by oxidant release from in vivo-stimulated granulocytes. Circumstantial data supporting the concept of indiscrete margination after thermal injury have been reported by Yurt and Pruitt.¹⁷ This phenomenon has been implicated in the development of organ failure after injury.^{18,19} This concept suggests that attempts to alter PMN function after injury should be aimed at preventing in vivo activation, which may restore chemotactic activity, rather than further stimulating neutrophil oxidative activity, which could result in increased organ injury.

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